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14. ABSTRACT <u>Abstract:</u> This study was designed to understand the role and implications of MUC16 cytoplasmic tail in breast cancer pathogenesis. We would like to update our findings with respect to it since our last report submission. One important question towards this end was whether MUC16 indeed undergoes cleavage, which was addressed using a dual-epitope tagging (N-ter FLAG and C-ter HA). We demonstrated that cleavage of MUC16 could be taking place in the membrane proximal region (twelve amino acids), however deletion of these twelve amino acids partially abrogated the cleavage. Further, Circular Dichroism (CD spectra) analysis using the bacterially purified protein showed an increased alpha helical nature of the protein at acidic pH (5.8). In addition, we demonstrate that the cellular location for the cleavage is Golgi apparatus and the acidic pH in the Golgi is critical for the cleavage. We therefore believe that structural changes brought about by the acidic pH in the Golgi is probably the major reason for the cleavage of MUC16 C-ter and this could be auto-proteolytic in nature. Besides, we verified our preliminary observation of N-glycosylation using site directed mutagenesis approach and we demonstrate that MUC16 C-ter undergoes N-glycosylation and this is critical for the stability of the protein. Here we have demonstrated that, MUC16 C-ter undergoes pH dependent cleavage in the Golgi apparatus and it takes place in the membrane proximal region of the protein. It undergoes Ubiquitylation and N-glycosylation, which are required for its stability. We are furthering our studies to understand the functional and mechanistic insight into its role in cancer pathogenesis.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8

Introduction: Breast cancer (BC) is a major health problem worldwide and is the second leading cause of cancer related deaths in American women with 232,340 and 39,620 estimated new cases and death respectively in 2013 (1). This accounts for 14% of all cancer related deaths among women in USA (1). Therefore, efforts should be undertaken to improve the survival of breast cancer patients with respect to early diagnosis and therapeutic intervention. Mucins are high molecular weight glycoproteins expressed by various epithelial cells and deregulated mucin production is increasingly being recognized as the hallmark feature of the inflammatory and neoplastic disorders of several organs including breast (2, 7). The ability of the membrane-bound mucins to sense the extracellular environment and carryout the outside-in signaling (2) and their differential expression in cancer compared to the normal tissues (particularly in breast and pancreas) makes these interesting candidates for study with respect to diagnosis and therapeutics. The C-terminal regions of these trans membrane mucins have been shown to be most vital with respect to their signaling functions, MUC1 being the most widely characterized (3). In this study we are characterizing the MUC16 C-terminal region. MUC16 is a type-I membrane-bound mucin with a heavily glycosylated N-terminal domain, a transmembrane (TM) region and a 32-residue cytoplasmic tail (CT) domain. Our recent studies demonstrate that normal breast do not express MUC16; however, its expression progressively increases in primary and metastatic breast cancer (7). In addition, MUC16 knock down in breast cancer (BC) cells resulted in significant reduction in the growth and tumorigenic properties of MDAMB231 cells (7). In the present study, to understand the mechanistic involvement of MUC16 in BC, we have focused on its C-terminal region. Although it has been predicted that MUC16 undergoes cleavage/shedding in the extracellular SEA domain(s) by proteases such as MMP7 and neutrophil elastase, the exact nature and site of cleavage and the fate of the membrane bound portion (C-terminal) is not entirely understood. The post-cleavage C-ter region is interesting due to presence of (a) stretch of polybasic aminoacids, the site of interaction for cytoskeletal proteins like Ezrin/Radixin/Moesin, (b) potential nuclear localization signal (bioinformatics prediction) which might facilitate its nuclear localization with associated transcription factors modulating the transcription of various target genes and (c) several potential serine, threonine and tyrosine phosphorylation sites facilitating its interaction(s) with proteins influencing oncogenic signaling pathways. *Therefore, we hypothesized that the oncogenic potential of MUC16 is in part, mediated by the potential involvement of C-terminal domain of MUC16.* Two specific aims were laid out for the study.

Aim 1: To investigate the MUC16 cytoplasmic tail domain in tumor growth and metastasis of BC cells. Aim 2: To determine the mechanism(s) of MUC16CT mediated BC progression by identifying potential phosphorylation sites and interacting partners of MUC16CT.

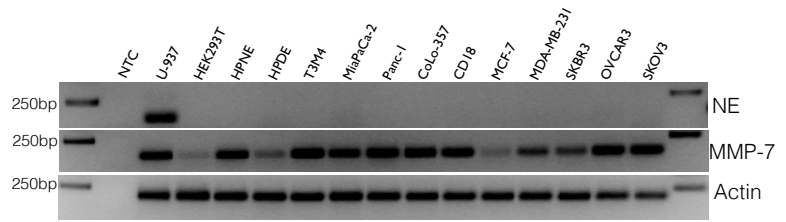


Fig 1A. Expression analysis of neutrophil elastase (NE) and MMP-7 in various cell lines by reverse transcriptase PCR (RT-PCR)

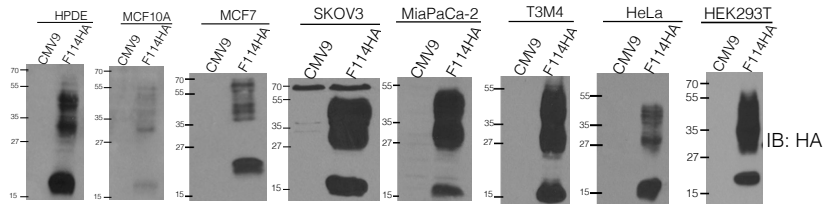


Fig 1B. Cleavage of MUC16 C-ter in various cell lines. These cells were either transiently or stably transfected with F114HA and cell lysates were probed with anti-HA antibody.

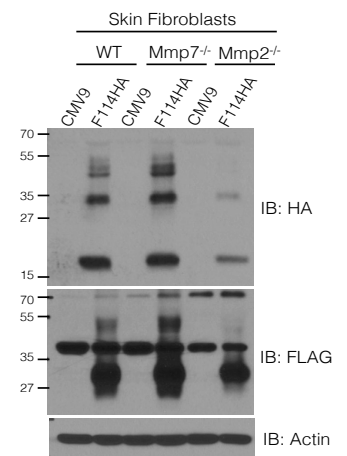


Fig 1C. Mmp7 KO and Mmp2 KO skin fibroblasts were transfected with F114HA construct and assessed for the cleavage using anti-HA and anti-FLAG antibody.

Body: (a) Cleavage of MUC16: As mentioned in our previous report, we narrowed down the cleavage of MUC16 to be taking place in the membrane proximal 12 amino acids. In an effort to identify any proteases responsible for the cleavage of MUC16, we sought to investigate the role of Neutrophil elastase (NE) and MMP-7 as has been proposed in literature (4). However, NE was not expressed in any of the cell lines examined by PCR (**FIG 1A**), (U937 is used as a positive control), but the cleavage of MUC16 was found to be universal in all the cell lines examined (**FIG 1B**). Since, none of the cell line makes NE, and FBS does not contain any neutrophil elastase, we rule out the involvement of NE in the cleavage of MUC16 C-ter. On the other hand, all the cell lines examined expressed MMP-7 (**Fig 1A**), making it an ideal candidate for the cleavage. To test the hypothesis, we established skin fibroblasts from MMP7^{-/-}, MMP2^{-/-}

and WT mice, which were then transfected with F114HA construct to assess the effect on the cleavage. However, we did not observe any effect of MMP-7 or MMP-2 in the cleavage of MUC16 C-ter (**Fig 1C**). Therefore, we believe neither NE nor MMP-7 has any effect on the cleavage of MUC16 C-ter.

Further, we investigated the cellular localization of MUC16 cleavage i.e. spatial arrangement of MUC16 C-ter cleavage (whether in the endoplasmic reticulum (ER), Golgi apparatus or plasma membrane). To study this we pretreated HeLa cells with Brefeldin-A (BFA) for various period of time and then transfected F114HA construct and maintained the cells further in BFA for different time period. BFA prevents the transport from ER to Golgi and vice-versa by dissolution of Golgi membranes, which then merges with ER membranes. Removal of this drug can reversibly restore the Golgi apparatus. As shown in

Fig 2A, we found that cells pre treated with BFA were unable to cleave MUC16 and the removal of the drug again resulted in the cleavage. We therefore concluded that the cleavage of MUC16 is taking place in the Golgi, not at the ER or the plasma membrane. Since BFA treatment resulted in the dissolution of Golgi compartmentalization, we reasoned that the lower pH in the Golgi could be a factor in the cleavage of this protein. Therefore, we carried out the similar experiment with pretreatment of Bafilomycin A1 (a proton pump inhibitor) and NH₄Cl (a weak base), which would neutralize the acidic compartment such as Golgi. A

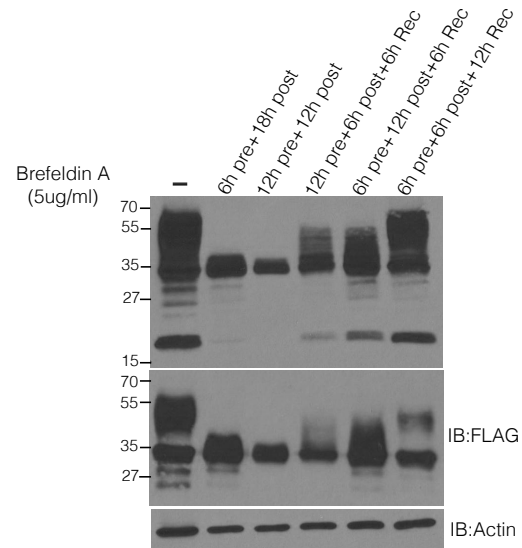


Fig 2A. Cleavage of MUC16 C-ter takes place in the Golgi. Pretreatment of HeLa cells with BFA followed by transfection with F114HA and post treatment for different time points followed by recovery.

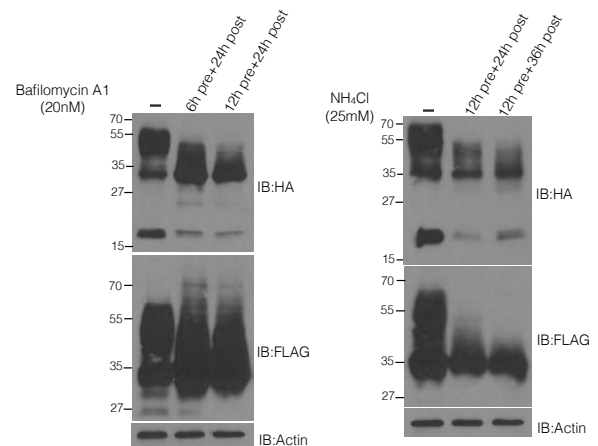


Fig 2B. Acidic pH is critical for the cleavage of MUC16 C-ter. Bafilomycin A1 and NH₄Cl were pretreated followed by transfection of F114HA and were treated post transfection. Cell lysates were probed with anti-HA and anti-FLAG antibodies and we observe that neutralizing the acidic pH severely affect the cleavage of MUC16 C-ter.

significant inhibition of cleavage of MUC16 C-ter was observed following Bafilomycin A1 and NH₄Cl treatment, suggesting the requirement for an acidic pH for the cleavage (**fig 2B**).

As mentioned in our previous report, alanine scan mutagenesis of the 12 extracellular amino acids did not result in abrogation/reduction of cleavage of MUC16 C-ter suggesting the cleavage may not be primary sequence dependent. Therefore, we generated a 12 amino acid deletion construct using F114HA, which did not result partial reduction of cleavage (**Fig 3A**), further supporting our hypothesis of requirement of a specific structure instead of the primary sequence for cleavage. Since, acidic pH in the Golgi was shown to be a prerequisite for the cleavage, we attempted to purify this protein from bacteria using Ni-NTA columns (His-tagged MUC16 C-ter 114 amino acid fragment). Though the protein was found to be insoluble, we could solubilize it using LPPG (lipid and detergent) buffer and carried out a circular dichroism (CD) analysis at two different pH i.e. pH=7.5 and pH=5.8. Following the CD analysis we observed a 5% increase in the alpha-helical nature of the MUC16 C-ter at acidic pH (5.8) compared to pH 7.5 (**Fig 3B**). Cleavage of MUC16 C-ter is taking place in almost every cell line we tried and is affected by neither neutrophil elastase nor MMP-7. In addition, we demonstrated that the site of cellular cleavage is Golgi and it is pH dependent. In addition circular dichroism analysis shows a structural change (increase in 5% alpha helical nature of the protein) under acidic pH compared to pH=7.5. We therefore believe that the cleavage of MUC16 C-ter could be autoproteolytic.

Significance: C-ter of MUC16 has been shown to be critical in mediating the metastatic nature of ovarian (5), breast and colon cancer cells (6), it is extremely important to understand the cleavage event, which will enable us to devise ways to target it.

(b) Post-translational modifications of MUC16 C-terminal region: In addition to the ubiquitylation as shown in our previous report, we had preliminary observation to suggest MUC16 C-ter undergoes N-glycosylation, which contributes to its higher molecular weight. To further ascertain the findings, we used site directed mutagenesis approach (**Fig 4**) to demonstrate that N-glycosylation in the extracellular region of MUC16 C-ter is responsible for the higher mol weight as well as the stability of MUC16 C-ter.

Significance: As demonstrated, the stability of MUC16 C-terminal region is dependent on the kind of post-translational modification it undergoes (Ubiquitylation and N-Glycosylation) and therefore understanding this is very crucial to understanding its function.

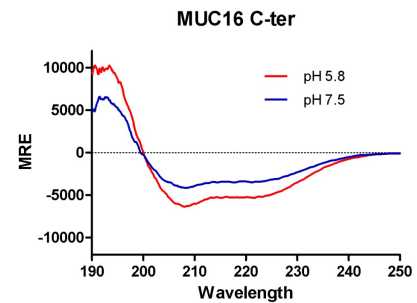
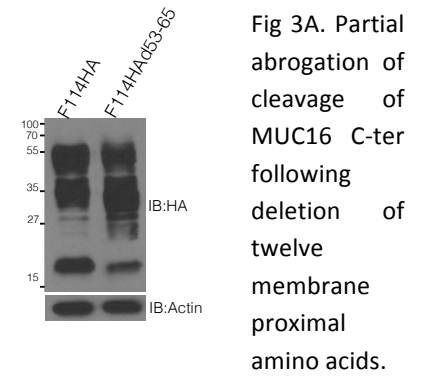


Fig 3B. Circular Dichroism (CD) analysis of MUC16 C-ter at pH=7.5 and pH=5.8. We observe an increase in the alpha helical content of MUC16 C-ter at acidic

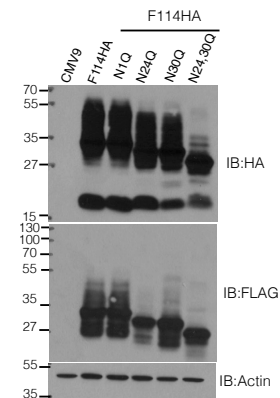


Fig 4. Site-directed mutagenesis of N-glycosylation sites (N to Q) confirms that MUC16 C-ter indeed undergoes N-glycosylation.

Key Research Accomplishments:

- (a) Cleavage of MUC16 is taking place in the membrane proximal region.
- (b) Cellular location for cleavage of MUC16 C-ter is the Golgi apparatus.
- (c) The cleavage of MUC16 C-ter is pH dependent and possibly autoproteolytic
- (d) MUC16-CT undergoes N-glycosylation, which is also responsible for its stability.

Reportable Outcomes:

Currently we are in the process of finalizing the draft our work for the publication.

Conclusions and Future Directions: Previous studies from our lab (Lakshmanan et al., 2012) have demonstrated that MUC16 plays a critical role in the breast cancer pathogenesis we focused our attention on the C-terminal region of MUC16 to be a major player in this process. In order to understand this, the first and most important question we wanted to address was whether it undergoes cleavage or not. By using dual epitope tagging we have convincingly demonstrated that it indeed undergoes cleavage. Though we have not found the exact site of cleavage, we believe that the cleavage of MUC16 is not sequence specific; instead it could be determined by the structural alteration induced by acidic pH in the Golgi. As our understanding of human diseases grow deeper, we realize the fact that post-translational modification(s) of protein are extremely important for its function. Here we demonstrate that MUC16CT undergoes ubiquitylation and N-glycosylation, which determines the stability of it, may turn out to be the most critical factor for its functions. Further experimental validations are being carried out to understand the role of MUC16-CT in neoplastic diseases with particular emphasis on breast cancer pathogenesis.

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